Somatic Embryogenesis and Plantlet Regeneration in Callus Cultures Derived from Mature Zygotic Embryos of Slash Pine[1]

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Abstract White, translucent, and mucilaginous embryogenic callus was initiated in cultured mature zygotic embryo explants of two different seed sources of slash pine(*Pinus elliottii*) on several culture media containing auxin and cytokinin. Somatic proembryos were induced on media containing 2,4-D and BA. Maturation was successfully achieved on medium supplemented with ABA. Somatic embryos germinated into regeneration plantlets on DCR medium containing activated charcoal. Histological observation and scanning electron microscopic observation showed that proembryos derived from embryonal suspensor mass(ESM) were formed on the surface or the inside of embryogenic callus, and the proliferation of proembryos was mainly from cleavage polyembryos.

Key Words: *Pinus elliottii*; Somatic embryogenesis, Plantet regeneration

Introduction

Somatic embryogenesis has a great potential for rapid propagation of superior conifer species. The first report on somatic embryogenesis and plantlet regeneration in conifer species was achieved in *Picea abies* from immature zygotic embryos in 1985[1], significant progress has been made in achieving somatic embryogenesis and plantlet regeneration in explants of forest trees[2-3]. Recently, somatic embryos were readily induced in embryogenic callus from mature zygotic embryo explants of *Picea abies*[4], *Picea sitchensis*[5], and *Pinus lambertiana*[6]. However, somatic embryogenesis and plantlet regeneration in Pinus was mainly from embryogenic callus derived from immature zygotic embryo explants [7-9]. The purpose of this study was to induce somatic embryogenesis from mature zygotic embryo explants of slash pine(*Pinus elliottii* Engelm).This is the first report of the induction of somatic embryogenesis in slash pine from mature zygotic embryos.

Materials and Methods

Plant material

Mature cones of two different seed sources of slash pine (*Pinus elliottii*)were collected from Shaoyang Seed Orchard (SS) and Zhangjiajie Seed Orchard (ZS), Hunan province, China, in October, 1994 to 1996.All cones were stored in plastic bags at 4 °C for 1 month to 18 months. Before dissection of mature zygotic embryos from megegametophyte, seeds were disinfected by immersion in 70% ethanol for 30 s and in 0.1% mercuric chloride for 15 min, following by 5 rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from the seeds and placed horizontally on a solidified callus induction medium in flasks. Each treatment comprised 50 explants and experiment was repeated three imes.

Somatic embryogenesis and plantlet regeneration protocol

Callus induction medium consisted of DCR medium[6] supplemented with 10 mg/L 2,4-D and 2mg/L BA. The induction frequency of callus from mature zygotic embryos was determined after six weeks on induction medium. Callus was subcultured on the callus proliferation medium with 2,4-D and BA decreased 5 times. After three weeks of proliferation culture, white translucent glossy mucilaginous callus was selected to use for differentiation culture. The differentiation medium con-

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sisted of DCR medium containing growth regulators (2,4-D, IAA, IBA, and ABA) either singly or in combination with BA(2 mg/L). Six weeks after culturing, the differentiation frequency of late stage somatic embryos (including procotyledonary, cotyledonary, and postcotyledonary somatic embryos) and mean number of somatic embryos per gramme callus were counted. Each treatment was replicated three times, and each replicate consisted of 50 callus cultures, averaging 1.8 g per tissue. Late stage somatic embryos were transferred to DCR medium containing 0.5-2.5 g/L activated charcoal. Six weeks after culturing, the frequency of plantlet regeneration was determined Each treatment was replicated three times , and each replicate consisted of 30 somatic embryos. In the callus induction, callus proiiferation, somatic embryo differentiation and germination medium, the concentration of sucrose, casein hydrolysate, glutamine, and agar were 30 g/L, 500mg/L, 500 mg/L, and 7 g/L, respectively. The PH adjusted to 5.8 with IN KOH or IN HCH prior to autoclaving at 121 °C for 18 min. Callus induction and proliferation took place in the darkness at 23 ±1°C and somatic embryo differentiation and germination took place in the light with 12 h photoperiod at 25±1°C

Histological and scanning electron microscopical observation

For hestological studies, callus cultures with somatic embryos was fixed in formalim, acetic acid, and ethylalcohol(1:1:18, v/v) for 24h, dehydrated through graded series of ethylalcohol and tertiary butyl alcohol and embedded in paraffin (58-60 °C), serial section of 8um thickness were cut with rotary microtone and stained with 1% aqueous crystal violet solution. For scanning electron microscopical observation, callus cultures were processed as described for scanning electron microscopy[3].

Results and Discussion

Callus induction and proliferation

10-20 days after culturing callus development has begun in the mature zygotic embryos of two different seed sources (SS and ZS)on DCR medium containing 2,4-D and BA. As the callus started to proliferate, three types of calli could be distinguished: white translucent glossy mucilaginous (Fig.1), light yellowish loose globular, and light green loose globular. Jain et al[4] also observed these three types of calli in cultured Norway spruce mature embryos. White translucent glossy mucilaginous callus was initiated from 21.3% and 15.6% of the SS and SZ mature zygotic embryos on DCR callus induction medium with 2,4-D and BA. White translucent glossy mucilaginous callus was embryogenic, and was selected to proliferate on DCR callus proliferation medium with 2,4-D and BA decreased 5 times.

Differentiation and development of somatic embryos

Six weeks after proliferation culture, white translucent glossy mucilaginous callus was transferred to differentiation medium with different growth regulators (2,4-D, IAA, IBA, and ABA) either singly or in combination with BA (2mg/L). The results showed that the highest differentiation frequency of 81.7% and 59.3% of the SS and ZS were observed on DCR medium containing 0.5 mg/L 2,4-D and 2 mg/L BA, respectively. The highest mean number of somatic embryos per gramme of callus of 16.2 and 9.7 of the SS and ZS were obtained on the same medium, respectively. Somatic embryos formed at much lower frequency, when IAA, IBA, or ABA were used at 0.5 mg/L(Table 1). Abscise acid(2-8 mg/L) alone was advantageous for the formation of postcotyledonary somatic embryos. The role of ABA in the maturation of somatic embryos was also described in *Pinus caribaea*[9].

Table 1. The effect of growth regulators on somatic embryo differentiation of slash pine

Growth regulators	Differentiation frequency of somatic embryos(%)		Mean number of somatic embryos per g of callus	
	SS	ZS	SS	ZS
0.5 mg/L 2,4-D + 2 mg/L BA	81.7 ± 2.7	59.3 ± 2.9	16.2 ± 1.6	9.7 ± 1.2
0.5 mg/L IAA + 2 mg/L BA	27.1 ± 1.5	21.7 ± 1.2	7.3 ± 1.1	5.2 ± 0.7
0.5 mg/L IBA + 2 mg/L BA	19.6 ± 1.2	18.5 ± 0.8	5.1 ± 1.0	3.3 ± 0.6
0.5 mg/L ABA + 2 mg/L BA	13.2 ± 1.4	12.3 ± 0.9	3.2 ± 0.5	2.5 ± 0.5
2 mg/L ABA	9.8 ± 1.2	8.6 ± 1.1	2.7 ± 0.8	1.9 ± 0.7
4 mg/L ABA	16.3 ± 1.6	12.5 ± 1.2	4.6 ± 1.1	3.1 ± 0.9
8 mg/L ABA	11.4 ± 0.9	10.6 ± 0.9	3.7 ± 0.9	2.9 ± 1.0

*SS: Shaoyang slash pine seeds; ZS: Zhangjiajie slash pine seeds. Values represent the mean ± SD.

The observation of scanning electron microscope showed that the proembryos derived from embryonal suspensor mass on the surface of embryogenic callus were formed in the third week of differentiation culture, and these proembryos had white head (Fig.2). Histological observation showed that the proembryos derived from embryonal suspensor mass in the inside of embryogenic callus had a embryonal head and a suspensor

(Fig.3). Cleavage polyembryos were also observed in the inside of embryogenic callus (Fig. 4). the development of somatic embryos is unsynchronous. Embryonal suspensor mass and proembryos were simultaneously observed in the suspension cultures of embryogenic callus (Fig. 5).

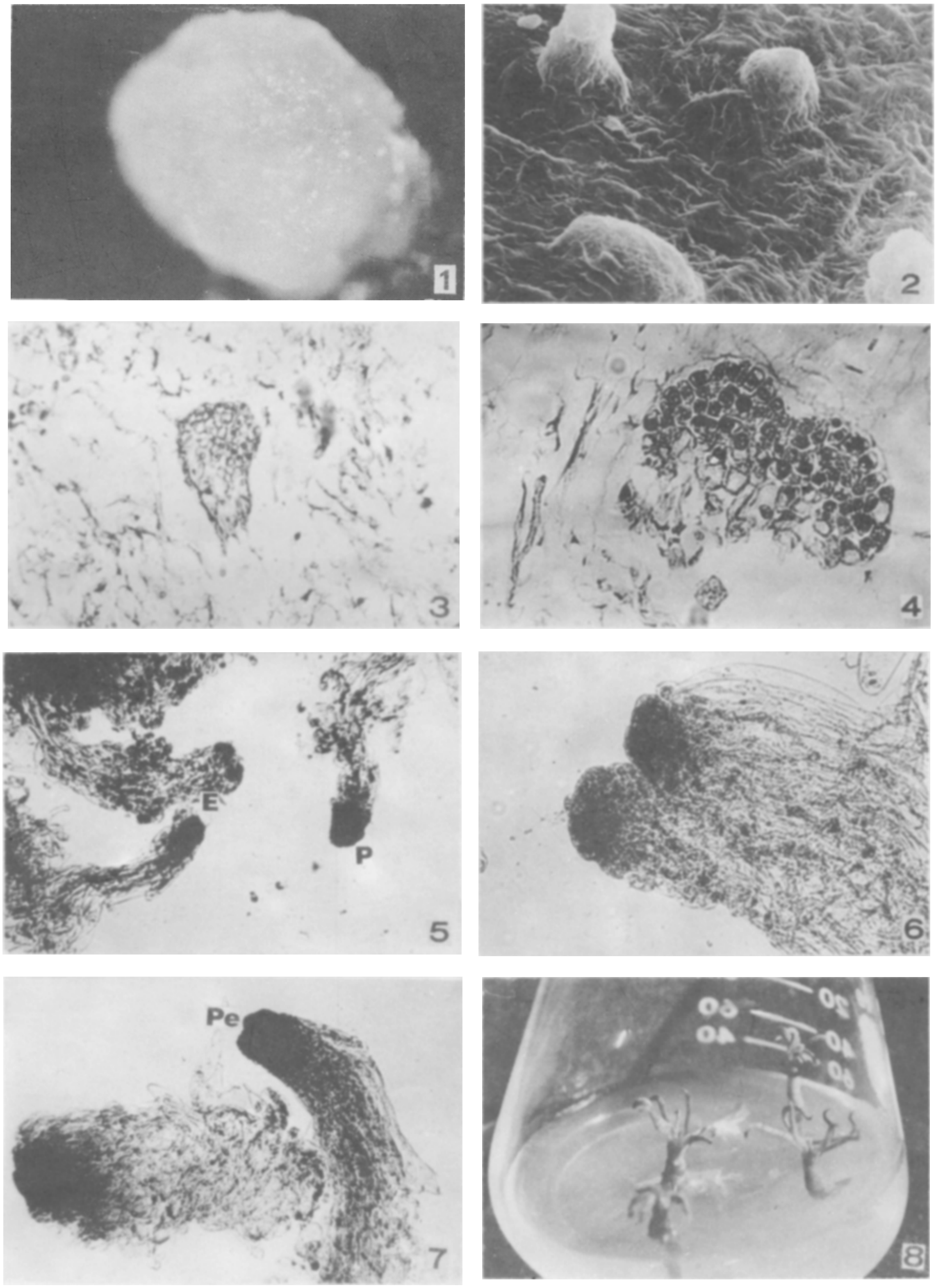

Fig.1. Embrogenic callus ×5; Fig.2. Proembryos on the surface of embryogeneic callus ×80; Fig. 3. Proembryos in inside of embryogeneic callus ×80; Fig. 4. Cleavage polyembryos ×160; Fig. 5. Embryonal suspensor mass (E) and proembryo(p) ×40; Fig. 6. Cleavage polyembryos with separate heads ×80; Fig. 7. Procotyledonary somatic embryos (pe). ×20; Fig. 8. Regeneration plantlets ×1.5

And cleavage polyembryos had separated heads in the sixth week of differentiation culture (Fig. 6). Procothledonary somatic embryos were formed in the ninth week of differentiation culture (Fig.7).

Somatic embryo germination and plantlet regeneration

somatic embryos were transferred to germination medium consisted of DCR medium containing 0.5-2.5 g/L activated charcoal. Six weeks after culturing, the results showed that the highest germination frequency of 56.2% and 34.1% of the SS and ZS was obtained on the DCR medium with 2g/L activated charcoal (Table 2). The important role of activated charcoal in the germination of somatic embryos of conifers was also proved in some research[2,7]. Regeneration plantlets from somatic embryos of slash pine have been subcultured for nine weeks on germination medium (Fig. 8). At present we are further investigating the process of transferred plantlets to soil.

Table 2. Effects of activated charcoal on the germination frequency(%) of somatic embryos in slash pine

Levels of activated charcoal(g/L)	Seed sources	
	SS	ZS
0.5	37.5 ± 1.6	28.7 ± 1.8
1.0	41.2 ± 2.1	29.1 ± 1.1
1.5	48.3 ± 1.7	31.4 ± 1.3
2.0	56.2 ± 2.9	34.1 ± 1.7
2.5	47.1 ± 2.5	30.5 ± 1.9

*SS: Shaoyang slash pine seeds; ZS: Zhangjiajie slash pine seeds. Values represent the mean ± S.D.

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